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Mutation and Enhanced Virus Transformation of Cultured Hamster Cells by Exposure to Gaseous Ethylene Oxide

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Mutation and Enhanced Virus Transformation of Cultured Hamster Cells by Exposure to Gaseous Ethylene Oxide

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Ethylene oxide is a classical mutagen and a carcinogen based on evidence from studies in experimental animals. It is widely distributed in industrial, research, hospital, and food environments. In an effort to explore the use of newly developed methods for exposing mammalian cells to gaseous or volatile mutagens/carcinogens, Chinese hamster V79 cells were treated for 2 hr with gaseous ethylene oxide, in sealed treatment chambers, and assayed for survival and mutagenic response by analysis of induced resistance to 6-thioguanine or ouabain. Significant numbers of mutants were produced at both genetic markers by 1,250–7,500 ppm ethylene oxide. Similarly, primary Syrian hamster embryo cells were treated for 2 or 20 hr with gaseous ethylene oxide in sealed treatment chambers and subsequently assayed for survival and increased sensitivity to SA7 virus transformation. Treatment concentrations extended from toxic to several nontoxic concentrations. After 2-hr ethylene oxide treatment at 625–2,500 ppm a significant enhancement of virus transformation was observed. At 20 hr after treatment no enhancement was observed. Treatment of hamster cells with ethylene oxide in both bioassay systems yielded concentration-related, quantitative results.

Key words: in vitro, gene mutation, virus transformation, V79 cells, ethylene oxide, Syrian hamster embryo cells

INTRODUCTION

Ethylene oxide (EO) is a large-production-volume chemical with exposure widely distributed in industrial, research, hospital, and food environments. EO is used in the synthesis of commercial chemicals such as ethylene glycol, acrylonitrile,

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and nonionic surfactants [Fishbein, 1969] and is used as a fumigant, agricultural fungicide, and sterilizing agent. It is an alkylating agent that interacts with both proteins [Calleman et al, 1978] and nucleic acids [Ehrenberg, 1979] and is mutagenic in bacteria, *Drosophila*, barley [Ehrenberg, 1979], mammalian cells [Tan et al, 1981], and rodents [Cumming and Michaud, 1979; Embree et al, 1977; Generoso et al, 1980].

With regard to the ability of EO to induce cancer, Hogstedt et al [1979] have suggested that EO may have caused excess mortality and cancer incidence in exposed human populations. In carcinogenicity studies in experimental animals, Dunkelberg [1979] has reported a low incidence of injection site sarcomas in NMRI mice, while other studies in mice have been inconclusive [IARC, 1976]. However, multiple evidence indicates that EO is carcinogenic in rats. Sprague-Dawley rats treated with EO by gavage in lifetime studies exhibited a large incidence of squamous cell carcinomas of the stomach [Dunkelberg, 1982]. In two lifetime inhalation studies with EO in Fischer 344 rats, increased incidences of mononuclear cell leukemia, peritoneal mesothelioma, and primary brain tumors were observed [Lynch et al, 1984; Snellings et al, 1984]. Teratogenic effects produced by this compound are weak in mice [Kimmel and Labrode, 1979] and negative in rats [Snellings et al, 1979].

Methods for treating mammalian cells with gases or vapors of volatilized liquids have been developed in our laboratory. These techniques have allowed the evaluation of the genotoxic effects of chlorinated methanes and ethanes and formaldehyde [Hatch et al, 1982a, 1983a,b] using chemical enhancement of virus transformation in primary Syrian hamster embryo (SHE) cells. This study was designed to evaluate the use of this exposure methodology for detecting gene mutation and chemical enhancement of virus transformation utilizing the classical mutagen EO.

This report describes the results of treating hamster cells with gaseous EO and reports its genotoxic effects utilizing quantitative bioassays for both mutation and enhanced DNA virus transformation.

MATERIALS AND METHODS

Chemicals

EO was purchased from Matheson Gas Co., East Rutherford, NJ, at a concentration of 10% in nitrogen. The EO-nitrogen mixture was prepurified to gravimetric standards and was ordered immediately prior to use. N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) and benzo(a)pyrene were purchased from Sigma Chemical Co., St. Louis, MO. They were first dissolved in acetone and then added to the medium to give a final concentration of 0.5% acetone in the medium. Ouabain and 6-thioguanine (6-TG) were purchased from Sigma Chemical Co., St. Louis, MO. Ouabain was dissolved in boiling water prior to dissolution in medium; 6-TG was dissolved in 1 N sodium hydroxide prior to dissolution in medium.

Cells

V79 Chinese hamster lung cells [Ford and Yerganian, 1958] were used to detect induced gene mutations. The V79 cells were routinely subcultured twice weekly at a seeding density of 8×10^4 cells in 15 ml of medium per 100-mm Petri dish. Williams' medium E (Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal bovine serum [FBS] (Sterile Systems, Inc., Logan, UT), 0.22 gm% sodium

bicarbonate (Mallinckrodt, Inc., Paris, KY), and 2 mM L-glutamine (Leon Labs, St. Louis, MO) was used for maintenance of cells.

Primary SHE cells were prepared by trypsinization of eviscerated and decapitated embryos (Charles River Laboratories, Boston, MA) after 13–14 days of gestation. Cells were resuspended in modified Dulbecco's medium (MDM) [Casto, 1973] supplemented with 10% FBS and 0.22 gm% sodium bicarbonate. Approximately 5×10^6 cells in 5 ml of medium were plated into 60-mm glass (Corning Pyrex) Petri dishes and incubated in a 5% CO_2 atmosphere at 37°C. Total cell number after 3 days ranged from 4 to 6×10^6 cells/plate.

Treatment of V79 and SHE Cells With EO in Modular Chambers

V79 cells were seeded into 60-mm glass Petri dishes (10^6/dish) in 5 ml of Williams' medium E supplemented with 10% FBS, 0.22 gm% sodium bicarbonate, and 2 mM L-glutamine 16 hr prior to treatment. Each dish contained approximately 1.5×10^6 cells per dish at the time of treatment. For treatment, the medium was removed and replaced with 1 ml of medium supplemented with 7.5 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid) buffer in place of sodium bicarbonate. The cells from three dishes at each concentration were combined after treatment prior to reseeding or subculture. Primary SHE cells cultured for 72 hr in 60-mm glass Petri dishes were fed with 1 ml of medium supplemented with 7.5 mM HEPES buffer immediately prior to treatment. Two dishes each containing about 4–6 $\times 10^6$ cells per dish were treated at each EO concentration. The cells were then reseeded to determine enhancement of transformation as previously described [Hatch et al, 1983b].

The cells were placed in polycarbonate treatment chambers of 4.6-liter volume (Billups-Rothenberg, Flow Labs, McLean, VA) and 10 ml of sterile water was added to the bottom of each chamber to ensure adequate humidification. EO-nitrogen was premixed with appropriate volumes of nitrogen and oxygen using precalibrated rotometers and mass flowmeters to give the desired final exposure concentrations of EO. Final oxygen concentration was 20%. Following introduction of varying concentrations of EO, the sealed chambers containing the cells were placed onto rocker platforms at 37°C. The chambers were tilted to provide a sufficient inclination (10°) and rocked at an appropriate rate (4×/min), so that a portion of the cell monolayer was always in contact with EO. Gases were exhausted after a 2-hr (V79) or 2- and 20-hr (SHE) exposure, and the cells were removed from the chambers. Untreated control cells were exposed to a mixture of 80% nitrogen/20% oxygen in the chambers.

Quantitation of Mutation to Ouabain and 6-TG Resistance

After exposure, the medium was changed back to complete Williams' medium E supplemented with 10% FBS. Twenty-four hours later, the V79 cells were dissociated with 0.05% ethylenediaminetetraacetic acid (EDTA) (Mallinckrodt, Inc., St. Louis, MO). The cells were then seeded into 60-mm Petri dishes to determine plating efficiency and the frequency of ouabain-resistant (Oua^r) mutants. The methods for selection of Oua^r mutants of V79 cells have previously been described by Langenbach et al [1978] and have been used with modification. Plating efficiency was determined 6–7 days after seeding 200 cells/4 ml medium/Petri dish by enumerating the number of colonies in five Giemsa-stained dishes/concentration. The frequency of Oua^r mutants was determined 13–15 days after seeding 2×10^5 cells/4 ml medium/Petri

dish with 40 dishes/concentration. Ouabain was added in 1 ml of medium to a final concentration of 1 mM 1 day after seeding. The mutation frequency for Oua^r was calculated per 10^6 survivors, based on the number of mutant colonies, the plating efficiency, and number of cells seeded for mutant selection.

To determine the frequency of 6-TG-resistant (TG^r) mutants, V79 cells from the same population used to determine ouabain resistance were seeded at 5×10^6 cells/dish into two 100-mm Petri dishes. Ninety-six hours after seeding, the cells from the two dishes were dissociated, combined, and reseeded into 60-mm Petri dishes to determine plating efficiency and the frequency of TG^r mutants; 6-TG was added in 1 ml of medium to a final concentration of 5 μg/ml, 2 days after seeding the cells for mutant selection. Plating efficiency was determined as described above. The methods for quantifying the frequency of induced TG^r mutants have been previously reported [Langenbach et al, 1981]. The frequency of TG^r mutants was determined 10–12 days after seeding 2×10^4 cells/4 ml medium/Petri dish from at least 40 dishes/concentration. The mutation frequency for TG^r was calculated per 10^6 survivors, based on the number of mutant colonies, the plating efficiency, and the number of cells seeded for mutant selection.

Quantitation of Enhanced SA7 Virus Transformation

After EO treatment, SHE cells were rinsed with complete MDM and inoculated with SA7 virus. Adenovirus transformation procedures have been previously reported [Casto, 1968, 1973; Hatch et al, 1982b, 1983a,b]. SA7 virus was adsorbed for 3 hr ($3–4 \times 10^7$ SA7 plaque-forming units per 60-mm Petri dish containing $5–7 \times 10^6$ cells). The virus-inoculated cells were removed with trypsin, centrifuged, and resuspended to 10^6 cells/ml in MDM with 10% FBS and 0.11 gm% sodium bicarbonate. After vigorous mixing, 2×10^5 cells were plated onto each of ten 60-mm plastic Petri dishes (Lux, Newbury, CA), followed by 3 ml of MDM with 10% FBS and 0.11 gm% sodium bicarbonate. Two days later, the medium was changed to 3 ml of MDM with 0.1 mM $CaCl_2$ [Freeman et al, 1967; Casto, 1968], 10% FBS, and 0.22 gm% sodium bicarbonate. After 6 days, transformation assay plates were overlaid with 3 ml of the above medium containing Bactoagar (0.3 gm/100 ml). At weekly intervals, 3 ml of additional agar medium was added. Final focus counts were made 25–30 days from the beginning of the experiment.

Survival was determined by plating treated SHE cells at 700 cells/dish (5 dishes/concentration) in 3 ml of MDM with 10% FBS and 0.11 gm% sodium bicarbonate. Two to 3 days later, 3 ml of medium with twice the amount of sodium bicarbonate was added to each plate, and after 8–9 days of total incubation, the cell colonies were fixed in 10% buffered formalin and stained with 0.02% crystal violet. The cloning efficiency of virus-inoculated cells under these conditions was usually 8–15%. The number of colonies in five plates seeded with treated cells was divided by the number of colonies in five plates seeded with control cells to find the surviving fraction of treated cells.

Detailed methods for determining the frequency of transformation and enhancement ratios using data from actual enhancement experiments have been previously described [Casto et al, 1973; Hatch and Anderson, 1985; Hatch et al, 1985]. Enhancement was expressed as the ratio between the transformation frequency of treated cells and the transformation frequency of control cells. Statistical significance was determined using a table of ratios [Casto et al, 1973] derived from the Lorenz table

[Lorenz, 1962] that is based on the Poisson distribution. The increased transformation frequency (TF) was considered statistically significant at the 5% or 1% confidence level if the enhancement ratio exceeded the appropriate value obtained from the Lorenz table.

RESULTS

Mutagenic Effect of Gaseous EO in V79 Cells in Modular Chambers

The mutagenic activity of EO was determined by resistance of V79 cells to ouabain and 6-TG using four concentrations of EO ranging from 625 to 7,500 ppm in two experiments (Table I). The cytotoxicity measurements for the calculation of the mutation frequencies were determined after 24 hr or 5 days of recovery after treatment and probably do not reflect the absolute cytotoxicity induced by EO. The data from experiment 1 indicate that the mutation frequency increased from six Oua^r mutants per 10^6 survivors at 625 ppm EO to 128 Oua^r mutants per 10^6 survivors at 7,500 ppm EO with a relative survival of 13% at the highest concentration. In experiment 2, cytotoxicity was evident at lower concentrations compared to experiment 1. However, the absolute mutation frequency for ouabain resistance was higher in experiment 2 at 1,250 ppm EO. In experiment 1, the mutation frequency for resistance to 6-TG increased from nine mutants per 10^6 survivors at 625 ppm EO to 283 mutants per 10^6 survivors at 7,500 ppm EO. The absolute mutation frequency for 6-TG resistance was increased in experiment 2 compared to experiment 1 to 478 TG^r mutants per 10^6 survivors at 5,000 ppm. In both experiments for both genetic markers clear concentration-response relationships were evident. The spontaneous mutation frequencies were 1–4 Oua^r and 3–15 TG^r mutants per 10^6 survivors determined in nonchemically treated V79 cells exposed to 80% nitrogen/20% oxygen in the chambers. MNNG, which has been reported previously to be mutagenic to V79 cells, was used as a positive control to characterize the sensitivity of the cells to a strong mutagen. The

TABLE I. Induction of Oua^r and TG^r Mutants by 2-hr Treatment of V79 Cells With Gaseous EO

	Oua^r				TG^r			
Treatment concentration (ppm)	Plating efficiency[a], %		Oua^r mutants/10^6 survivors		Plating efficiency[b], %		TG^r mutants/10^6 survivors	
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
7,500	6	0	128 (20/13)[c]	—[d]	30	0	283 (68/40)	—[d]
5,000	14	0.2	41 (29/25)	—[d] (13/40)[e]	48	39	52 (20/40)	478 (149/40)
1,250	31	14	6 (14/40)	29 (32/40)	58	40	19 (9/40)	96 (31/40)
625	53	ND	6 (25/37)	ND	41	ND	9 (3/40)	ND
0	47	23	1 (2/40)	4 (13/80)	47	50	3 (1/40)	15 (12/80)
MNNG[f] 0.5 μg/ml	24	15	103 (198/40)	82 (98/40)	38	44	401 (122/40)	607 (214/40)

[a]Determined from plates receiving 200 cells 24 hr after exposure.
[b]Determined from plates receiving 200 cells 5 days after exposure.
[c]Values in parenthesis are numbers of mutant colonies scored/numbers of plates scored.
[d]No mutants recovered.
[e]Mutation frequency not calculated due to low plating efficiency for ouabain selection.
[f]V79 cells were treated in HEPES buffer with MNNG for 4 hr. The treated cells were not placed in the exposure chamber.

MNNG-treated cells were not treated in exposure chambers but were concurrently treated with the EO-exposed cells. MNNG induced 82–103 Oua^r mutants and 401–608 TG^r mutants per 10^6 survivors at a concentration of 0.5 μg/ml. The use of HEPES buffer and the rocking procedure probably reduced the plating efficiency of V79 cells in these studies compared to studies performed in plastic Petri dishes using Williams' E medium [Langenbach et al, 1981].

Enhancement of Virus Transformation in SHE Cells by EO in Modular Chambers

EO-exposed SHE cells were examined for their ability to produce more virus-transformed colonies than 80% nitrogen/20% oxygen-exposed SHE cells when both groups were subsequently infected with SA7 adenovirus.

SHE cells were exposed for 2 hr with gaseous EO in three experiments (Table II). After this treatment the two highest treatment concentrations (2,500 and 5,000 ppm) produced significant cytotoxicity (0–62% survival) as determined by the survival assay. Lower exposure concentrations were less cytotoxic. EO treatment resulted in enhanced virus transformation in all three experiments in a concentration-dependent manner; 1,250 ppm and 2,500 ppm EO produced statistically significant ($P < .05$) increased enhancement ratios of 1.9–2.9 and 4.6–9.9, respectively. A large absolute increase in the number of viral foci was observed at 1,250 ppm and 2,500 ppm EO in two of the three experiments. SHE cells treated for 2 or 20 hr (not in chambers) with the concurrent positive control chemical benzo(a)pyrene exhibited significantly increased ($P < .01$) enhancement ratios: 1.8–2.8 (Table II).

SHE cells exposed to EO for a longer treatment time, 20 hr, at concentrations of 1,250, 2,500, and 5,000 ppm and then infected with SA7 virus did not produce any more foci than cells exposed to 80% nitrogen/20% oxygen and virus. The results of this experiment were: EO concentration (ppm), % survival, number of SA7 foci/2

TABLE II. Enhancement of Virus Transformation of SHE Cells by 2-hr Pretreatment With EO

Treatment concentration (ppm)	% survival[a]			No. SA7 Foci[b]			Enhancement Ratio[c]		
	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3
5,000	0	0	0	0	0	0	0	0	0
2,500	11	62	19	47	115	30	*9.9*	*4.6*	*7.2*
1,250	80	82	76	99	77	31	*2.9*	*2.4*	1.9*
625	112	87	58	52	73	33	1.1	*2.1*	*2.6*
0	100	100	100	43	40	22	1.0	1.0	1.0
Benzo(a)pyrene[d] 0.5 μg/ml	93	91	87	94	100	54	*1.8*[e]	*2.8*	*2.8*

[a]Determined from plates receiving 700 cells. Cloning efficiencies (%) of control cells were Exp 1, 6.1, Exp 2, 9.9, Exp 3, 7.1.

[b]Number of foci from 2×10^6 plated cells.

[c]The enhancement ratio was determined by dividing the transformation frequency of treated cells by that obtained from control cells. Italicized numbers indicate significance at the 1% level. *Significant at the 5% level.

[d]SHE cells were treated in HEPES buffer with benzo(a)pyrene for 20 hr (Exp 1) or 2 hr (Exps 2, 3). The treated cells were not placed in the exposure chambers.

[e]Control data taken from the concurrent 20-hr EO experiment in which there were 56 SA7 foci/2×10^6 plated cells in the untreated control group.

$\times 10^6$ plated cells, and enhancement ratio: 0, 100, 56, 1.0; 1,250, 100, 59, 1.1; 2,500, 98, 52, 0.95; 5,000, 0, 0, 0.

DISCUSSION

We report the successful quantitative evaluation of EO for both mutation and enhancement of virus transformation utilizing treatment methodologies for exposing mammalian cells to gaseous chemicals.

The use of V79 cells to evaluate the induction of mutational events by diverse agents has been thoroughly reviewed [Bradley et al, 1981]. Chinese hamster V79 cells were treated with EO and assayed for mutagenic response by analysis of the induced resistance to 6-TG and ouabain. Significant numbers of mutants were produced using gaseous exposure techniques and could be readily detected by either genetic marker. The mutation frequencies produced by EO are approximately 32–94 and 7–128 times the spontaneous background levels for TG^r and Oua^r, respectively. More than a threefold increase is generally accepted as significant [Bradley et al, 1981]. Treatment concentrations for mutagenic effects were approximately two- to fourfold higher than those needed for enhancement of virus transformation.

The mutagenicity data reported here for gaseous EO-exposed V79 cells is similar in frequency for TG^r to that of Tan et al [1981], who reported concentration-dependent mutagenicity and cytotoxicity of EO in the CHO/HGPRT system using sealed flasks. They concluded EO was functioning as a direct-acting agent independent of metabolic activation. Zamora et al [1983] have also reported EO-induced mutagenesis (TG^r) in CHO cells using both dynamic and static exposure systems. The results reported here are consistent with those of Tan et al [1981] and Zamora et al [1983] and demonstrate significant mutagenic effects of EO in V79 cells utilizing modular exposure chambers as well as including an additional marker for mutation (Oua^r).

The virus enhancement assay reflects the capacity of a chemical to damage cell DNA by either direct or indirect means and provides a rapid mammalian cell bioassay system for screening of suspect genotoxic environmental chemicals. With regard to mechanism, it has been shown that carcinogen treatment results in an increased incorporation of virus DNA into cellular DNA [Casto et al, 1979] and that covalent B(a)P-DNA adducts are formed in SHE cells exposed to B(a)P [Theall et al, 1982]. The assay has been used to evaluate single chemicals as well as complex mixtures [Casto et al, 1981] and multiple agents [Hatch and Anderson, 1985]. Positive test results in this system are generally concordant with those obtained in bacterial and mammalian cell mutagenesis assays and in in vivo carcinogenesis assays [Casto, 1981; Hatch et al, 1982a, 1983b, 1985].

EO produced a significant enhancement of virus transformation at 1,200–2,500 ppm after 2 hr of treatment. The longer treatment time (20 hr) was not effective since EO gas concentrations probably decrease with time, presumably by adsorption and/or penetration of chamber walls and by degradation in the culture media. Such a decrease in chemical vapor concentrations in these treatment chambers has been documented by analysis of volatile aliphatic chlorinated hydrocarbons over increasing time as determined by gas chromatographic techniques [Hatch et al, 1982a, 1983b]. If the concentration of EO decreased with time to ineffective levels, then a longer treatment time might provide a period for cellular repair processes to remove DNA

damage. This hypothesis is supported by the observation with formaldehyde that virus enhancement does not occur if a 20-hr recovery period (before addition of virus) is allowed following a 2-hr treatment time [Hatch et al, 1983a]. Also, a correlation between enhancement of virus transformation and persistence of DNA damage of both alkylating agents and polycyclic hydrocarbons has been demonstrated [Casto et al, 1976].

The results presented here confirm and extend the previous in vitro studies on the mutagenic and potentially carcinogenic effects of EO in mammalian cells. The demonstrated ability of EO to interact with a variety of cellular macromolecules without the requirement for metabolic activation and its nonspecificity with regard to inducing effects in bacteria, *Drosophila*, barley, mammalian cells, and rodents suggest EO to be a potential environmental hazard.

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